Controlled delivery of antimicrobial gallium ions from phosphate-based glasses

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Abstract

Gallium-doped phosphate-based glasses (PBGs) have been recently shown to have antibacterial activity. However, the delivery of gallium ions from these glasses can be improved by altering the calcium ion concentration to control the degradation rate of the glasses. In the present study, the effect of increasing calcium content in novel gallium (Ga_2O_3)-doped PBGs on the susceptibility of Pseudomonas aeruginosa is examined. The lack of new antibiotics in development makes gallium-doped PBG potentially a highly promising new therapeutic agent. The results show that an increase in calcium content (14, 15, and 16 mol% CaO) cause a decrease in degradation rate (17.6, 13.5 and 7.3 $\mu g.mm^{-2}.h^{-1}$), gallium ion release and antimicrobial activity against planktonic P. aeruginosa. The most potent glass composition (containing 14 mol% CaO) was then evaluated for its ability to prevent the growth of biofilms of P. aeruginosa. Gallium release was found to reduce biofilm growth of P. aeruginosa with a maximum effect (0.86 Log_{10} CFU reduction compared to Ga_2O_3-free glasses) after 48h. Analysis of the biofilms by confocal microscopy confirmed the anti-biofilm effect of these glasses as it showed both viable and non-viable bacteria on the glass surface. Results of the solubility and ion release studies show that this glass system is suitable for controlled delivery of Ga^{3+}. ^{71}Ga NMR and Ga K-edge XANES measurements indicate that the gallium is octahedrally coordinated by oxygen atoms in all samples. The results presented here suggest that PBGs may be useful in controlled drug delivery applications, to deliver gallium ions in order to prevent infections due to P. aeruginosa biofilms.

Key words: Glass, Drug delivery, Microbiology, Antimicrobial, Biofilm

1. Introduction

Advances in medicine and surgery have led to increasing reliance on a variety of medical devices. However, the non-shedding surfaces of medical devices, such as catheters, frequently become colonised by members of the indigenous microbiota and opportunistic pathogens such as Pseudomonas aeruginosa which can cause hospital-acquired infections (HAIs). Many of the diseases caused by P. aeruginosa (such as airway infections in cystic fibrosis (CF) patients, chronic wound and sinus infections) appear to be associated with biofilm formation and are responsible for significant mortality [1-4]. Biofilm formation occurs as a result of a sequence of events: microbial surface attachment, cell proliferation, matrix production and detachment [5]. Biofilm-associated bacteria show a decreased susceptibility to antibiotics [6], disinfectants [7] and clearance by host defences [3, 8]. A recent study has found that Ga^{3+} ions inhibit P. aeruginosa growth and biofilm formation in vitro by decreasing bacterial Fe uptake and interfering with Fe signalling via the transcriptional regulator pvdS [9]. Other studies have demonstrated that gallium is effective against the organisms causing tuberculosis [10] and malaria [11] in human beings, and in the treatment of pneumonia due to Rhodococcus equi in foals [12]. Further to this, we have recently shown that Ga_2O_3-doped phosphate-based glasses (PBGs) containing Ga^{3+} exhibit a potent antibacterial effect against planktonic bacteria including P. aeruginosa, methicillin-resistant Staphylococcus aureus and Clostridium difficile [13]. However, bacteria grown planktonically are known to be far more susceptible to antibacterial agents compared to their biofilm counterpart [14].

Ga_2O_3-doped PBGs are durable materials which can act as a unique system for the delivery of gallium ions in a controlled way [13]. Ions incorporated into the glass structures are not a separate phase, and thus their rate of release is defined by the overall degradation rate of the glass. In the past copper and silver have been incorporated into PBGs and have then been used

as wound dressings to prevent infections [15] and also to control urinary tract infections in patients needing long-term indwelling catheters [15, 16]. However, there is an underlying need to improve the properties of existing biomaterials due to the incidence of HAIs, which often lead to revision surgery, and the growing resistance to antibiotics exhibited by these bacteria [17]. Despite the recent increase in the number of reported HAIs, there are very few new antibacterial drugs with an entirely new mechanism of action that has been introduced in the past three decades or are present in the advanced stages of development [18]. Our recent work showed the potential of Ga_2O_3–doped PBGs as a novel drug delivery system in combating bacteria associated with HAIs, especially P. aeruginosa [13]. However, in that study, it was found that increasing the gallium content of the glasses decreased the rate of degradation and subsequent release of gallium ions which highlighted the need to improve the controlled delivery of Ga^{3+} for antimicrobial applications.

The aim of the study reported here is to investigate the effect of increasing calcium content in Ga_2O_3-doped PBGs on their structure, properties and antibacterial activity against both planktonic cells and biofilms of P. aeruginosa.

2. Materials and Methods

2.1.Preparation of Ga_2O_3-doped PBGs

PBGs were produced using NaH_2PO_4 (BDH, ≥98%), P_2O_5 (Sigma, ≥97%), and $CaCO_3$ (BDH,≥98.5%). For the preperation of gallium-containing PBGs, Ga_2O_3 (Sigma, 99.99%) was also used as shown in Table I. The required amount of each reagent was weighed and added to a Pt/10%Rh crucible (Johnson Matthey, Royston, UK). The crucible was then placed in a preheated furnace at 1100°C for 1 hour. The molten glass was then poured into graphite moulds, which had been preheated to 350°C. The glass samples were allowed to cool to room

temperature, and the resulting glass rods cut into discs by using a rotary diamond saw (Testbourne Ltd, Basingstoke, UK). Density measurements were conducted in triplicate on samples using the Archimedes' Principle.

Gallium-doped glasses of general composition $(CaO)_x(Na_2O)_{52-x}(P_2O_5)_{45}(Ga_2O_3)_3$, where x = 14, 15 and 16, hereafter given the abbreviations C14, C15 and C16 respectively, were prepared along with a sample containing no gallium, hereafter given the abbreviation Ga0, of composition $(CaO)_{16}(Na_2O)_{36}(P_2O_5)_{45}$

2.2.Degradation study

Ga_2O_3-doped PBG rods (5 mm diameter and 2 mm thickness) with different CaO contents were put in plastic containers, filled with 50 ml of deionised water (pH 7±0.5), and placed in an incubator at 37 °C. At various time points (6, 24, 48, 72 and 120h), the three disks were taken out of their respective containers, and excess moisture removed by blotting the samples dry with tissue prior to weighing them. All the disks were put into a fresh solution of deionised water and placed back into the incubator. To obtain the rate of mass loss, the initial weight (M_0) of each sample was measured as well as the mass at time t (M_t) to give a mass loss per unit area thus: mass loss=$(M_0–M_t)/A$, where A is the surface area (mm^2). The measurements were carried out in triplicate, and the weight loss per unit area plotted against time. The slope of this graph gives a degradation rate value in units of $mg.mm^{-2}.h^{-1}$, determined by fitting a straight line of the form y = mx.

2.3. pH measurements

The pH measurements of the medium in which the glass disks had been soaked were taken at each time point (6, 24, 48, 72 and 120h) using a Hanna Instruments pH 211 Microprocessor

pH meter (BDH, UK) with an attached glass combination pH electrode (BDH, UK). The pH electrode was calibrated using pH calibration standards (Colourkey Buffer Solutions, BDH, UK).

Both dissolution studies and standards, for ion release study, were prepared using high purity water. This was obtained from a PURELAB UHQ-PS system (Elga Labwater, UK) with a purity level of 18·2 MΩcm^{-1} resistivity.

2.4. Ion release study

Ion release studies were simultaneously conducted, and the medium was analysed for Na^+ and Ca^{2+} using ion chromatography (Dionex, UK). ICP-MS (inductively coupled plasma mass spectrometry, Spectromass 2000 by SPECTRO) was used to determine the amount of both gallium and phosphorus ions released from all glass compositions at the previously mentioned time points. The instrument was calibrated for the concentration range 0.1-1000 ppb by mixing single element standards obtained from Sigma and diluting in ultra pure water.

2.5. Effect of increasing calcium concentration on planktonic P. aeruginosa growth

P. aeruginosa (PA01) cells were inoculated into 10 mL of nutrient broth and incubated overnight at 37 °C with 200 rpm agitation in an orbital shaker (Stuart Scientific, UK). The overnight cultures were used to inoculate 5 mL volume of phosphate buffer saline (PBS; Oxoid) to a standardized optical density of 0.03 at a wavelength of 600 nm (OD_{600}). Ga_2O_3-doped PBGs disks of 5 mm diameter and 2 mm thickness were added to each tube, with the gallium-free disk (Ga0) used as a control. The tubes were then incubated at 37 °C. At various time intervals (1, 12 and 24 h) serial dilutions of the suspensions were carried out in PBS. 50 μl volumes of the suspension and each dilution were spread onto MacConkey agar (Oxoid,

Basingstoke, UK) plates. The plates were then incubated aerobically at 30°C for 48 h. For each type of disc, viable counts (colony forming units; CFUs) were conducted in triplicate.

2.6.Effect of Ga_2O_3-doped PBGs on viability of biofilms

2.6.1. Constant Depth Film Fermentor (CDFF) study

A CDFF (University College Cardiff, Cardiff, UK), described previously by Mulligan et al. [19], was used for the production of biofilms. The CDFF contains a stainless steel turntable which can hold up to 15 polytetrafluoroethylene (PTFE) pans; with each PTFE pan holding 5 PTFE plugs. Discs, 5 mm in diameter, were placed on each plug and recessed to a depth of 300 μm. The PTFE pans were then inserted so that they were flush with the turntable. A cylindrical glass vessel and two stainless steel end plates encase the turntable. The top plate contains an air inlet port, to which two 0.2 μm Hepa-vent air filters (Fisher Scientific, UK) were attached. It also contains three media inlet ports. Incoming medium (in this case 1%TSB) drips onto the rotating turntable and is distributed over the PTFE pans by two scraper blades. The scraper blades also serve to maintain the biofilms on the discs at the required depth, equal to the depth of the recess. The bottom plate contains a medium outlet port. The CDFF was sterilized in a hot air oven, using a temperature of 160 °C for 1 h. During all experiments, the CDFF was incubated at 37 °C. The turntable rotated at a speed of 3 rpm.

2.6.2.Viable counts

At various time intervals, pans were removed aseptically from the CDFF. Each pan was washed with 10 ml of PBS. Discs containing P. aeruginosa biofilms were placed in 1 ml of PBS and vortexed for 1 min. to remove the attached biofilms and to disperse them in the suspension. Serial dilutions of the suspensions were carried out in PBS. 25 μl volumes of the suspension and each dilution were spread onto MacConkey Agar (Oxoid) plates. The plates

were then incubated aerobically at 37 °C for 48 h. For each type of disc, viable counts (colony forming units; CFUs) were conducted in triplicate.

2.6.3.Confocal laser scanning microscopy (CLSM)

A viewing solution was first prepared containing 8 ml of PBS together with 2 µl each of components A and B of BacLight™ LIVE/DEAD stain (Invitrogen, UK). The biofilm-coated discs were placed into a small cell-culture dish (Bibby Sterilin Ltd, Stone,UK), and covered with the viewing solution and the stains allowed to develop in the dark for 10 min. The biofilms were then examined using a microscope (Olympus BX51 microscope) which incorporated a Bio-Rad Radiance 2100 laser scanning system and LUMPlanFI 40x water lens. Two-channel (viable 'Live'/nonviable 'Dead') confocal image stacks were collected in 8-bit colour depth at a resolution of 1024×1024 pixels. The z-axis step size was typically 0.6 µm, however this was optimised for each image stack depending upon the total depth of the sample.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) was used to compare mean viable counts, following arcsinh transformation of data. When a significant difference was detected, a Tukey test was conducted to find which values were different (GraphPad Software; San Diego, USA.).

2.8. Thermal and Structural analysis of the Ga_2O_3-doped PBGs

2.8.1. Density Measurements

Density measurements were conducted in triplicate using Archimedes' Principle, on an analytical balance (Mettler Toledo, UK) with an attached density kit. Due to the soluble nature of the glasses investigated, ethanol was used as the displaced liquid for these measurements. The density of the glasses (ρ) were obtained employing equation (1),

$$\rho = \left(\frac{M_{dry}}{M_{dry} - M_{wet}} \right) \times \rho_{liquid}$$ ----------Equation (1)

where M_{dry} and M_{wet} are the masses of sample in air and liquid respectively, and ρ_{liquid} is density of ethanol at room temperature.

2.8.2.Differential Thermal Analysis

Differential Thermal Analysis (DTA) was carried out using a Setaram Differential Thermal Analyser (Setaram, France) on powdered glass samples of approximately 60 mg. Three main thermal parameters were measured; T_g, crystallisation temperature (T_c), and melting temperature (T_m). A temperature ramp from ambient up to 1000 °C at a rate of 6.7 °C.min^{-1} was used under nitrogen purge; an empty platinum crucible was used as a reference. The data were baseline corrected by carrying out a blank run and subtracting this from the original data [20, 21].

2.9. NMR Analysis

^{23}Na magic angle spinning (MAS) NMR experiments were conducted using a 3.2 mm diameter rotor spinning at 20 kHz. Spectra were acquired using a Bruker Avance II^{+} spectrometer attached to a 14.1 T magnet (^{23}Na Larmor frequency 158.7 MHz). Aqueous NaCl was used as a reference, with the sharp resonance from this set to 0 ppm. The liquid 90° pulse length was determined as 2.5 µs, although a much shorter pulse length (0.5 µs) was used on the solid samples. A one-pulse sequence was used, with a pre-acquisition delay of 4.5 µs and a recycle delay of 5 seconds. Around 100 scans were acquired for each experiment. . Certain ^{23}Na spectra were also recorded at 7.05 T under similar conditions.

^{31}P MAS NMR experiments were conducted using a 4 mm diameter rotor spinning at 10 – 12.5 kHz. Spectra were acquired using a Chemagnetics Infinity Plus spectrometer attached to

a 7.05 T magnet (^{31}P Larmor frequency 121.5 MHz). $NH_4H_2PO_4$ was used as a secondary reference compound, the signal from this set to 0.9 ppm (relative to 85% H_3PO_4 at 0 ppm). A pulse length of 1.5 μs was used (corresponding to a ~30° tip angle), with a pre-acquisition delay of 7.5 μs and a recycle delay of 5 seconds. Typically, 80 scans were acquired for each spectrum.

^{71}Ga MAS NMR experiments were conducted at 14.1 T (^{71}Ga Larmor frequency 183.0 MHz) using a Bruker Avance II spectrometer and a 3.2 mm rotor, spinning at approximately 18 kHz. A one-pulse sequence was used with a pulse length of 0.75 μs corresponding to a tip angle of ~30°, a pre-acquisition delay of 4.5 μs and a recycle delay of 2 seconds. Spectra were referenced to a saturated aqueous solution of gallium (III) nitrate at 0 ppm. Experiment times varied from 2 hours for the sample containing the most gallium, to 16 hours for the sample containing the least.

The recycle delays were sufficient to produce fully relaxed, quantitative spectra. All spectra were processed using TOPSPIN 2.0 or Spinsight and fitted using either dmfit2007 [22] or QuadFit .[23]

2.10. Ga K-edge XANES spectroscopy

Ga K-edge X-ray absorption near-edge structure (XANES) measurements were made at room temperature on Station 16.5 at the SRS, Daresbury Laboratory, UK, with a ring energy of 2 GeV and a stored current of 150-250 mA. The spectra were recorded in transmission mode using a double crystal Si(220) monochromator (d = 1.92 Å) and ionisation chambers to detect the incident and transmitted beam intensities. Finely-ground samples were diluted in polyethylene (Aldrich, spectrophotometric grade) and pressed into pellets to give a

satisfactory edge jump and total absorption. An encapsulated gallium foil and a third ionisation chamber were placed after the sample to allow an absorption spectrum of the foil to be collected simultaneously for the purpose of calibration of the energy scale. The energy scale was defined by assigning the point of maximum gradient on the absorption edge from the Ga foil to 10367 eV.

XANES spectra were collected from 50 eV below to 175 eV above the Ga K-edge in order to allow accurate background subtraction. A fine energy step of 0.4 eV was used around the edge. The data processing comprised conversion of the data to absorption versus energy, calibration of the energy scale, removal of the pre-edge background by straight-line fitting and removal of the post-edge background by fitting with a polynomial. All the spectra were normalised to have an edge-step of 1. As well as the data from the Ga_2O_3-doped PBGs, spectra were also collected from a series of crystalline reference materials containing Ga^{3+} ions in well-defined coordination geometries: quartz α-$GaPO_4$, β-Ga_2O_3, $Ga_2(SO_4)_3$ and $Ga(acac)_3$. The $Ga(acac)_3$ (Aldrich, 99.99%) and $Ga_2(SO_4)_3$ (Aldrich, 99.995%) were purchased commercially, whilst the quartz α-$GaPO_4$ and β-Ga_2O_3 were synthesized. The quartz α-$GaPO_4$ was prepared by precipitation from an aqueous mixture of $GaCl_3$ (Aldrich, 99.99%) and H_3PO_4 by the addition of NH_4OH. The product was separated by filtration, washed and dried before heating to 800 °C to remove ammonium and hydroxyl groups [24]. The β-Ga_2O_3 was prepared by calcination of Aldrich 99.99% Ga_2O_3 overnight at 1000 °C [25]. The gallium foil used for the calibration of the energy scale was prepared by hot-pressing Aldrich 99.99% Ga metal between two sheets of filter paper and laminating the resulting construct in plastic.

3. Results

3.1. Glass degradation, pH and ion release

3.1.1. Glass degradation

Degradation of Ga_2O_3-doped PBGs in aqueous solution leads to mass loss, pH variation and release of ions that yield key data to correlate with the in vitro antibacterial reaction experiments. As can be observed in figure 1a, the rate of mass loss decreased with increasing CaO content of the glasses. However, there were no perceptible differences in the profiles from the 15 and 16 mol% CaO compositions until 48 hours. The Ga_2O_3-free PBG was found to dissolve completely after 72 hours incubation. The dissolution rates, obtained by applying a line of best fit through the data, were 41.7, 17.6, 13.5 and 7.3 $\mu g.mm^{-2}.h^{-1}$ for the Ga0, C14, C15 and C16 compositions, respectively.

3.1.2. pH Analysis

The pH analysis revealed an increase in pH with decreasing CaO content (Figure 1b). The gallium free composition displayed the greatest increase in pH from an initial value of 7.0 to 8.6. The pH value for both the C14 and C15 samples remained close to neutral for the duration of the study. The hydrolysis of PBGs exhibits clear pH dependence; this is supported by other studies: Watanabe et al.[27] stated that the rate of hydrolysis of small ring cyclic trimeta- and tetrametaphosphates decreases in acidic solutions, and increases in basic solutions with an increase in the pH value for all solvents.

3.1.3. Ion release

The highest levels of Ca^{2+} (Figure 2a) and Na^{+} (Figure 2b) release were observed for the compositions with the highest dissolution rate, i.e. the Ga_2O_3-free and C14 glasses. The Ca^{2+} ion release data correlates well with the solubility data obtained. For the Ca^{2+} release profiles

(Figure 2a), the gallium-free composition released the greatest amount of ions. Also, compositions with higher sodium mol% released more Na^+ ions into solution, and it was directly proportional to the solubility values, suggesting that sodium ions were released into solution first.

Both Ca^{2+} (Figure 2a) and Na^+ (Figure 2b) ion release profiles showed clear differences between the compositions investigated, with a decrease in Ca^{2+} and Na^+ ion release seen with increasing CaO mol% content. The greatest Ca^{2+} and Na^+ release was seen for the Ga_2O_3-free composition and suggests that the presence of gallium ions (Ga^{3+}) led to the decrease of glass degradation and subsequent release of Ca^{2+} and Na^+ ions.

The release of phosphorus ions in this study did not follow a linear trend with time (Figure 2c).The rate of phosphorus ion release was higher for Ga_2O_3-free and C14 compositions and the Ga_2O_3-doped PBGs showed a decrease as the CaO content increased in the glasses, with C16 releasing the least amount of phosphorus. The use of ICP-MS has enabled the detection of the total amount of phosphorous ions, and this method is found to be superior to ion chromatography, where the lack of availability of standards restricts the complete detection of all the different phosphate species present.

As can be seen from Figure 2d, no Ga^{3+} was detected from the 0 mol% Ga_2O_3 composition as expected. Clear differences are observed between the Ga^{3+} release from the C14, C15 and C16 compositions. The C14 composition released the highest levels of Ga^{3+} ions, with the C16 composition releasing the least. The Ga and P release profiles reflected the degradation rate, as well as Na^+ and Ca^{2+} ion release of the glasses.

3.2. Antimicrobial analysis

3.2.1. Effect of increasing calcium content on planktonic P.aeruginosa

The effect of Ga_2O_3-doped PBGs with increasing CaO concentrations (C14, C15 and C16) on the viability of suspensions of P. aeruginosa is shown in Figure 3a. Ga_2O_3-free PBG samples were used as controls, and initial viable counts were conducted prior to addition of the PBGs to determine the number of viable bacteria present. Each point represents the $\log_{10}$ of the mean number of viable bacteria from three samples. Error bars represent standard deviations. The effect of Ga0, C14, C15 and C16 glasses on the pH of the bacterial suspensions was also measured and found to be 7.1±0.02. There was no statistically significant difference between the pHs of the various suspensions.

The C14, C15 and C16 glasses all showed statistically significant ($p = 0.0001$) reductions in the $\log_{10}$ of the mean number of viable cells compared to the control at 4, 12 and 24h (Figure 3a). Moreover, the $\log_{10}$ of the mean number of viable cells from C14-treated samples displayed maximum reduction throughout the study with the greatest effect at 24h compared to C15 and C16 ($p = .0001$). However, no statistically significant differences ($p \geq 0.870$) in the $\log_{10}$ of the mean number of viable cells were observed between C15 and C16 samples throughout the study. The reduction in the $\log_{10}$ of the mean number of viable cells observed for all samples, including controls, after 24h incubation suggested that the overall decrease in the viable count beyond this time point was largely attributable to nutrient depletion or accumulation of toxic end products of metabolism rather than to the presence of gallium.

However, bacteria grown planktonically are known to be far more susceptible to antibacterial agents compared to their biofilm counterpart [14]. Therefore, the most potent glass composition (C14) against planktonic growth was evaluated for its effect on the growth of biofilms of P. aeruginosa.

3.2.2. Effect of C14 glasses on the viable counts of *P. aeruginosa* in biofilms

Biofilm growth studies were conducted on C14 glasses with Ga0 glasses and Hydroxyapatite (HA) discs as controls (Figure 3b). Each point represents the $\log_{10}$ of the mean number of viable counts of three biofilms from one representative CDFF run. Error bars represent standard deviations. It should be noted that at least three runs for each experiment were performed to confirm the results obtained. The data were not pooled because slight differences in the inoculum produced differences in the absolute CFU numbers obtained. However, the relative differences found were very repeatable.

At 6 h, the C14 glass showed no significant difference in $\log_{10}$ of the mean number of viable cells compared to both the Ga0 and HA ($p \geq 0.07$) (Figure 3b). At 12 h, the $\log_{10}$ of the mean number of viable cells was significantly reduced for the C14 glass compared to both controls ($p = 0.0001$). This effect of the C14 glass on the viable count of the biofilms was also observed after 24 and 48h - the viable counts were significantly different from both the controls ($p \leq 0.001$). The greatest effect of gallium on biofilm growth was observed at 48 h (0.86 $\log_{10}$ CFU reduction compared to Ga0 glasses). However, at 72 h the $\log_{10}$ of the mean number of viable cells on C14 started to recover from the previous low at 48 h and showed no statistically significant difference compared to Ga0 glasses ($p = 0.1$).

3.2.3. Identification of live and dead P. aeruginosa cells using CLSM

The use of water immersion lenses and a liquid viewing medium (PBS) in the present study enabled the observation of biofilms in their natural hydrated state (Figure 3c). As seen in normal viewing of BacLight™ LIVE/DEAD stained images, used in the present study, the viable cells fluoresce green and the non-viable cells fluoresce blue (Figure 3c). The biofilms were submerged in the stains (at a relatively high concentration) for at least 15 min before the

CLSM scan. The molecular weights of the BacLight™ LIVE/DEAD stain components are similar (component A= 550–750 Da (proprietary information) and component B = 668.4 Da), and both have a net positive charge. It is therefore unlikely that there is any significant difference in their diffusion characteristics into biofilms. Regions of biofilm composed of viable and non-viable bacteria were observed on C14 glass samples (Figure 3c(ii)) as opposed to only viable bacteria on Ga0 samples (Figure 3c(i)). These observations confirmed that the reduction of biofilm growth was due to the presence of Ga in the glasses.

3.3. Structural analysis of the Ga_2O_3-doped PBGs

3.3.1. Density

Figure 4a shows the density (in $g.cm^{-3}$) of the four glasses studied. The results show that the addition of 3 mol% Ga causes a slight increase in density. This is to be expected since Ga^{3+} ions are significantly heavier and smaller than Ca^{2+} and Na^{+} ions. No significant variation in density was observed as a function of CaO content.

3.3.2. Thermal Analysis

Thermal analysis was carried out on the glass samples in order to measure glass transition temperatures (T_g) and investigate crystallisation and melting phenomena. Figure 4b shows DTA traces for the four glasses. From the DTA data it can be seen that the glass transition temperature (T_g) increased from 327.2 ± 1.2 ºC for 0 mol% Ga_2O_3 glass to 343.3 ± 2.0 ºC by the incorporation of 3 mol% Ga_2O_3. This increase in T_g could be attributed to the formation of more ionic cross-links between the phosphate glass chains by the incorporation of Ga_2O_3 into the glass network. However, maintaining the same Ga_2O_3 content while reducing CaO to 15 and 14 mol% showed no significant difference in the T_g compared to glasses with 16 mol% CaO. The DTA of 0 mol% Ga_2O_3 glass showed the presence of a single sharp crystallisation event at 454 °C and two melting peaks at 572 and 650 °C. Incorporation of 3 mol% Ga_2O_3

shifted the crystallisation temperature to 467 °C, and also the two melting peaks to 684 and 745 °C respectively. Maintaining the Ga_2O_3 at 3 mol% while reducing the CaO content to 15 mol% results in both the crystallisation and melting peaks shifting to lower temperatures. The crystallisation peak shifted to 457 °C, and the two melting peaks to 607 and 676 °C. Further reduction of CaO to 14 mol% produced a further shift of the crystallisation and melting peaks to even lower temperatures. The crystallisation temperature shifted to 450 °C while the melting peaks were shifted to 600 and 664 °C. This finding correlated well with the glass transition temperature data where a reduction in CaO contents resulted in a shift in the thermal parameters to lower temperatures.

3.3.3. NMR analysis

Solid-state NMR was the primary tool for structural characterisation of Ga_2O_3-doped PBGs and has already been proved to be extremely powerful in elucidating direct information on the speciation of the principal network former P_2O_5 and the gallium coordination environment in the glasses [13]. This structural information of the glasses is important to identify the optimal glass combination for the antibacterial applications. Figure 5a shows the ^{31}P MAS NMR spectra obtained from the glasses, with the horizontal scale expanded around the isotropic region so that only the centre-bands are shown. The connectivity of the phosphate network is commonly described by Q^n notation, where n refers to the number of bridging oxygens in the PO_4^{3-} group [28, 29]. Two peaks are clearly visible at chemical shifts of around −20 and −5 ppm, representing Q^2 and Q^1 phosphorous sites respectively, and figure 5b shows a fit of the C16 spectrum, including fitting of the spinning sidebands (the spinning sideband intensities were included in calculating the relative abundance of each Q^n species). No Q^3 or Q^0 sites were observed in any of the ^{31}P MAS NMR spectra recorded here. The peak positions, linewidths and relative abundances obtained by such fitting are presented in Table 2.

The ~25 % Q^1 phosphorus sites observed here are to be expected in these glasses since they are below the metaphosphate stoichiometry and therefore should contain chain-end groups. It can be seen from Table 2 that those glasses containing Ga show a slightly higher percentage of Q^2 phosphorus sites than the glass without Ga. This suggests that with the gallium present the network undergoes some slight rearrangement increasing the connectivity of the glasses. Both the Q^1 and Q^2 chemical shifts are more negative for the gallium-containing glasses, which is consistent with a previous study of a Ga_2O_3-containing phosphate glass [30].

Figure 5c(i) shows the overlaid ^{23}Na MAS NMR spectra obtained from the four samples. These spectra appear very similar in shape, with just a slight broadening of the line for the Ga-containing glasses. Figure 5c(ii) shows ^{23}Na MAS NMR spectra and their simulations for the 0 % Ga_2O_3 glass at two different fields using a Gaussian distribution in quadrupolar coupling constant χ_Q [29, 31, 32] to represent the variation in Na environments present in the sample due to disorder. These simulations yielded a mean value of χ_Q = (2.65 ± 0.15) MHz, a FWHM distribution in this parameter of (2.15 ± 0.15) MHz, and a chemical shift value of δ_{iso} = (−3 ± 0.5) ppm. The asymmetry parameter η_Q was kept as 0 for simplicity, although in reality a distribution in C_Q would likely mean a distribution in this, as well as the isotropic chemical shift. These parameters gave a good fit to all four ^{23}Na spectra, with only a slight decrease in the amount of line broadening for the 0 % Ga_2O_3 glass. These results imply that the extent of disorder of the sodium environment increases slightly with the addition of 3 % Ga_2O_3 but is not significantly affected by the differences in stoichiometries of the 3 % Ga_2O_3 samples studied here.

Figure 5d shows the ^{71}Ga MAS NMR spectra obtained from the three Ga-containing glasses, all obtained at 14.1 T. The spectra all show a lower signal/noise ratio than the ^{23}Na or ^{31}P

spectra due to the significantly smaller amount of gallium present in the sample, the lower natural abundance of the ^{71}Ga isotope (39.9 % compared with 100 % for both ^{23}Na and ^{31}P), and also the wider lineshape due to second-order quadrupolar broadening [29,31]. The relatively large linewidth in these spectra mean that the spinning sidebands lay very close to the centre-band, although they are not very clearly visible due to their amplitudes being comparable to that of the noise. Each spectrum shows a peak centred at approximately −50 ppm. The FWHM linewidth of this peak is approximately 100 ppm. A previous ^{71}Ga MAS NMR study on Ga_2O_3-Na_2O-P_2O_5 glasses identified a peak at −60 ppm associated with octahedrally coordinated gallium and one at 120 ppm due to tetrahedral gallium [30]. This suggests that the ^{71}Ga NMR peak observed here arises from octahedrally coordinated gallium. The presence of some tetrahedral gallium cannot be ruled out since a small peak may be present at the expected position for this coordination (around 100-200 ppm [29]), but is obscured by noise and spinning sidebands. However, the spectra do suggest that the gallium is present primarily in the octahedral coordination.

3.3.4. Ga K-edge XANES

XANES spectra was used to clarify any possible anomalies in antimicrobial effect of Ga_2O_3-doped PBGs due the local coordination environment around the gallium in the glasses. XANES spectra can give information on the coordination environment of a given probe atom, often by comparison of the spectra with those from materials containing the probe atom in a well-defined structural site. In this case, data was collected with higher energy resolution in the vicinity Ga K-edge from reference materials, (Figure 6a), and the Ga_2O_3-doped PBGs, C14, C15 and C16, (Figure 6b). The reference materials were chosen to have a range of gallium coordination environments: quartz α-$GaPO_4$ contains tetrahedrally coordinated gallium [24], β-Ga_2O_3 an equal mixture of tetrahedral and octahedral gallium [33], and

$Ga(acac)_3$ and $Ga_2(SO_4)_3$ both contain octahedral gallium [33]. Figure 6a shows that for the octahedrally-coordinated gallium a broad feature at ~10377 eV is observed, whilst for the tetrahedrally-coordinated gallium a distinct two-humped curve is seen in the same region with features centred at slightly higher and lower energy. The XANES spectrum from β-Ga_2O_3, the mixed-site material, contains features from those observed in both the single-site materials. Some variation is observed in the spectra from the compounds containing octahedral gallium: the main peak in the $Ga_2(SO_4)_3$ spectrum is broader and at slightly lower energy (~1 ev) than that in the spectrum from $Ga(acac)_3$. These differences can be explained by considering the level of distortion around the gallium sites in the two reference compounds. $Ga(acac)_3$ contains Ga^{3+} ions surrounded by a near-perfect octahedron of oxygen atoms [33], whereas in $Ga_2(SO_4)_3$ there are two gallium sites, both significantly distorted relative to ideal octahedral geometry [34]. These qualitative observations are in agreement with previous studies which demonstrated that different coordination sites could be distinguished using Ga K-edge XANES [25, 33, 35].

4. Discussion

This paper reports the effect of increasing calcium concentration in Ga_2O_3-doped PBGs on their antibacterial properties, physico-thermal properties, solubility, pH change and ion release. It is reported that most of the Q^1 species identified in the 45 mol% P_2O_5 glasses are phosphate dimers and therefore the packing density would be greater compared to 50 or 55 mol% P_2O_5 glasses [36]. Therefore the non-linear data obtained from the thermal and solubility analyses in the present study can be attributed to the packing density of the 45mol% P_2O_5 glass compositions. The solubility was seen to decrease with increasing CaO mol%. The observed reduction in dissolution rate associated with the increasing CaO content could be explained by the increase in the ionic strength of the leaching solution. Glass degradation has

been reported to consist of the three synergistic processes of ion exchange, hydration and finally hydrolysis of the phosphate chains while in solution [26]. As a result of ion exchange, a hydrated gel layer is usually formed on the glass surface and when it leaches into the surrounding medium it causes an increase in the ionic strength of the solution resulting in reduction of the dissolution rate. Both Ga0 and C14 compositions showed a gradual increase in pH with time, due to the depletion of H^+ in the solution that resulted from exchange with the Na^+ and Ca^{2+} ions released from the glass. The ion release profiles exhibited similar trends to the degradation rates obtained, and a decrease in the rate of phosphorus release was observed with increasing CaO content.

MAS NMR results suggest that as the sodium is replaced by calcium in the 3 mol% Ga_2O_3 glasses there is little change in the relative abundances of the Q^1 and Q^2 phosphorus sites. Both the Q^1 and Q^2 chemical shifts for the 3 mol% Ga-containing glasses move to a slightly more negative chemical shift as the sodium in the system is replaced by calcium. This is consistent with the observation reported by Brow et al.[37] that as the cation potential (charge to radius ratio) of the modifying cation increases, the phosphorous chemical shift becomes more negative (calcium has a larger cation potential than sodium). The Ga K-edge XANES spectra presented here from the gallium-doped PBGs (Figure 6b) exhibit no variation as a function of composition and show one broad feature that is similar in shape, intensity and magnitude to that observed for $Ga(acac)_3$ and $Ga_2(SO_4)_3$,(Figure 6a) suggesting, in agreement with the ^{71}Ga NMR results (Figure 5d), that the Ga^{3+} ions in all the glass samples are octahedrally coordinated. The position of the peak in the spectra from the glasses is very close to that observed in the $Ga_2(SO_4)_3$ spectrum. Also, in common with the $Ga_2(SO_4)_3$ spectrum, some asymmetry is noted in the peak in the glass spectra. Both of these observations suggest some degree of distortion around the gallium site in the glasses.

Our previous work suggested that Ga_2O_3-doped PBGs, with a P_2O_5 content of 45 mol % and CaO content of 16 mol %, are capable of broad-spectrum bactericidal activity against planktonic bacteria including P. aeruginosa [13]. In the present study, the sample containing 14 mol % CaO demonstrated the greatest antibacterial activity against planktonic P. aeruginosa and this composition also gave excellent long-term release of Ga^{3+} ions into the medium. Recently, an antimicrobial approach using Ga^{3+} was reported that targets bacterial Fe^{3+} metabolism by exploiting the chemical similarities between Fe^{3+} and Ga^{3+} [9]. Trivalent gallium is capable of interacting with:

1. the iron-dependent enzyme such as ribonucleotide reductase causing inhibition of DNA synthesis [38]
2. superoxide dismutase and catalase that protect against oxidant stress [39]
3. enzymes involved in oxidative phosphorylation such as cytochromes and others [9].

The ability of Ga^{3+} to interfere with iron-dependent enzymes also suggest that Ga^{3+} could act on several of the above said targets simultaneously [9] and hence mutation of a single intracellular target might not produce high-level Ga^{3+} resistance in subjected bacteria. However, in a biofilm environment, microbes exhibit reduced susceptibility to antimicrobial agents [14]. The results of this study have shown that the release of Ga^{3+} ions from the 14 mol% CaO sample can achieve a significant reduction in the growth of P. aeruginosa biofilms over at least a 48h period (Figure 3b). CLSM analysis confirmed the presence of both viable and non-viable bacteria on the surface of C14 glass samples (Figure 3c(ii)) confirming the ability of gallium to kill P. aeruginosa in biofilms.

5. Conclusions

Our findings suggest that these Ga_2O_3-doped PBGs, particularly the C14 composition, hold promise as antimicrobial agents and could offer some advantages over conventional

therapeutic agents. Firstly, Ga^{3+} is proposed to work by a completely different mechanism to conventional drugs [9] that will not lead to high-level Ga^{3+} resistance in subjected bacteria. Moreover, Fe^{3+} levels are so low in human tissues and the activity of Ga^{3+} is increased when Fe^{3+} is limited [9] will enhance the chances of Ga_2O_3-doped PBGs being more effective under physiological conditions than our in vitro test results presented here. Finally, the fact that, Ga^{3+} is approved by FDA for intravenous administration and the ever existing scarcity of new antibiotics in development make Ga_2O_3-doped PBGs a potentially highly promising new therapeutic agent against P. aeruginosa.

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Figure legends

Figure 1. Dissolution, determined by mass loss (a), and pH analysis (b) of Gallium free (Ga0) and gallium containing (C14, C15 and C16) PBGs as a function of time.

Figure 2. Cumulative ion release (a) calcium, (b) sodium, (c) phosphorous, and (d) gallium as a function of time Ga0, C14, C15 and C16 PBGs.

Figure 3. [a] The effect of Ga0, C14, C15 and C16 PBGs on the viability of suspensions of P. aeruginosa at 4, 12 and 24h of incubation. CFU= colony forming units (mean number of viable cells). **[b]** Log_{10} CFU/mm^2 of P. aeruginosa in biofilms formed on hydroxyapatite discs (HA), Ga0 and C14 PBGs. **[c]** CLSM images after 48h of P aeruginosa biofilms on (i) Ga0 and (ii) C14 PBGs. Viable (green) and non-viable (blue) bacteria.

Figure 4 [a] Density ($g.cm^{-3}$), **[b]** DTA trace of Ga0, C14, C15 and C16 PBGs as a function of CaO contents.

Figure 5[a]. ^{31}P MAS NMR spectra obtained Ga0, C14, C15 and C16 PBGs at 7.05 T. **[b]** The fit of the ^{31}P MAS NMR spectrum from the C16 PBG sample, including the spinning sidebands. **[c]** (i) Overlay of the ^{23}Na MAS NMR spectra from Ga0, C14, C15 and C16 PBGs at 7.05 T and (ii) simulation of the Ga0 PBG sample at multiple fields.**[d]** The ^{71}Ga MAS NMR spectra of the samples containing gallium, C14, C15 and C16 PBGs, at 14.1 T.

Figure 6. Ga K-edge XANES spectra from (a) crystalline reference materials: $Ga(acac)_3$ (solid line), $Ga_2(SO_4)_3$ (dashed line), β-Ga_2O_3 (dotted line) and quartz α-$GaPO_4$ (dashed-dotted line), and (b) Ga-doped PBGs: C14 (solid line), C15 (dashed line) and C16 (dotted line).

Tables

Table 1. Composition of phosphate-based glasses used in this study

Table 2. ^{31}P MAS NMR fit parameters for the Q^1 and Q^2 sites.

Table 1

Glass code	Glass code used in the text	Glass composition (mol %)			
		Calcium Oxide	Sodium Oxide	Phosphorous Pentoxide	Gallium Oxide
$Ca_{16}Na_{39}P_{45}$	Ga0	16	39	45	0
$Ca_{14}Na_{38}P_{45}Ga_3$	C14	14	38	45	3
$Ca_{15}\ Na_{37}P_{45}Ga_3$	C15	15	37	45	3
$Ca_{16}\ Na_{36}P_{45}Ga_3$	C16	16	36	45	3

Table 2.

Glass code	Q^2 Shift / ppm (± 0.1 ppm)	Q^2 Abundance / % (± 0.5 %)	Q^2 Linewidth / ppm (± 0.1 ppm)	Q^1 Shift / ppm (± 0.1 ppm)	Q^1 Abundance / % (± 0.5 %)	Q^2 Linewidth / ppm (± 0.1 ppm)
Ga0	−20.2	73.5	9.5	−3.6	26.5	8.5
C14	−20.6	75.9	9.3	−6.2	24.1	11.0
C15	−20.7	76.0	9.4	−6.3	24.0	10.7
C16	−20.8	75.2	9.7	−6.4	24.8	10.9